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PARTIAL MORPHOLOGICAL AND FUNCTIONAL CHARACTERIZATION OF THE CORPUS ALLATUM–CORPUS CARDIACUM COMPLEX FROM THE TWO-SPOTTED STINKBUG, *PERILLUS BIOCULATUS* (HEMIPTERA: PENTATOMIDAE)

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SUMMARY

Selected morphological and physiological properties of the corpus allatum (CA)–corpus cardiacum (CC) complex from the two-spotted stinkbug, *Perillus bioculatus* (Hemiptera: Pentatomidae), were studied. The CAs play an important role in insect physiology because of their production of the juvenile hormones (JHs), i.e., key hormones involved in development and reproduction. We found that the *P. bioculatus* CA–CC complex is present in two distinct morphological forms, the more frequently observed complex containing one "fused" CA between two CCs and the more rarely observed complex having one CA laterally attached to each CC. These complexes were tested for their ability to synthesize JH-like compounds. We found that the primary lipophilic compound synthesized by the CA–CCs migrated differently from JH III (a JH found in numerous insect species) when subjected to thin-layer chromatography. Furthermore, the synthesis of this compound is stimulated by 2*E*,6*E*-farnesol, a known precursor for JH III. These data indicate that the *P. bioculatus* CA–CC product has chemical properties similar to that of other (as of yet unidentified) hemipteran JHs. In addition, we found that the synthesis of this product is sensitive to pH and buffer type; minimally or not affected by the absence of the CC; expressed at similar levels in days 5–30 postemergent adults; and inhibited or decreased in adults reared under low temperature–short day conditions.

Key words: juvenile hormone; Heteroptera; farnesol; Colorado potato beetle; thin layer chromatography; diapause.

Insect corpora allata (CA) are endocrine tissues that form a complex with the corpora cardiaca (CC, neuroendocrine tissues), known as the CA–CC complex. The CAs produce juvenile hormones (JHs), which are generally known to be methyl epoxyfarnesoates and are important in development and reproduction (Wigglesworth, 1985; Englemann, 1990; Davey, 1997, 2000; Gilbert et al., 2000). The JHs having different structures have been identified from a variety of insect species (Wigglesworth, 1985; Englemann, 1990; Kotaki, 1997; Davey, 2000), with the structure of hemipteran JHs having not yet been identified (Davey, 1997, 2000; Kotaki, 1997). Our studies involved a hemipteran, the two-spotted stinkbug, *Perillus bioculatus* (Hemiptera: Pentatomidae), known to be an effective predator of the pest insect, the Colorado potato beetle, *Leptinotarsa decemlineata* (Biever and Chauvin, 1992; Hough-Goldstein and McPherson, 1996).

The CA activities are commonly studied by in vitro assays, which use organic solvents to specifically remove the JHs present in the incubation medium (Feyereisen and Tobe, 1981; Feyereisen, 1985). These assays have been used to study the rates and regulation of the synthetic products of the CAs (i.e., the JHs), as well as to assist in the elucidation of the structure of JH (Jones and Yin, 1989; Hodkova et al., 1996; Kotaki, 1996, 1997; Gilbert et al., 2000). The objectives of this study were to partially characterize selected morphological and functional features of the *P. bioculatus* CAs, as well as to gain initial insights into the regulation and properties of its JH-like product(s). In our assays, the addition of 2*E*,6*E*-farnesol ((*E*,*E*)-3,7,11-trimethyl-2,6,10-dodecatrien-1-ol) to the incubation medium was used to stimulate synthesis because it is a precursor of JH III. This hormone (JH III) has been shown to enhance the production of an important egg protein, vitellogenin, in *P. bioculatus* (Adams et al., 2002; T. A. Coudron and S. L. Brandt, data not shown). The 2*E*,6*E*-farnesol also has been shown to stimulate CA output in other hemipterans (Kotaki, 1996, 1997).

The CAs were removed from adult *P. bioculatus* that were reared in 16-h light at 26° C, 55% relative humidity (RH), and were primarily fed larvae of cabbage looper (*Trichoplusia ni*). On occasion, separate groups of *P. bioculatus* were fed larvae of Colorado potato beetle (*L. decemlineata*). No differences were observed in CA product formation between adults fed the different larval species (data not shown).

In *P. bioculatus*, the CA–CC complex is located adjacent to the dorsal aorta (Fig. 1), in close proximity to the brain. The CA is

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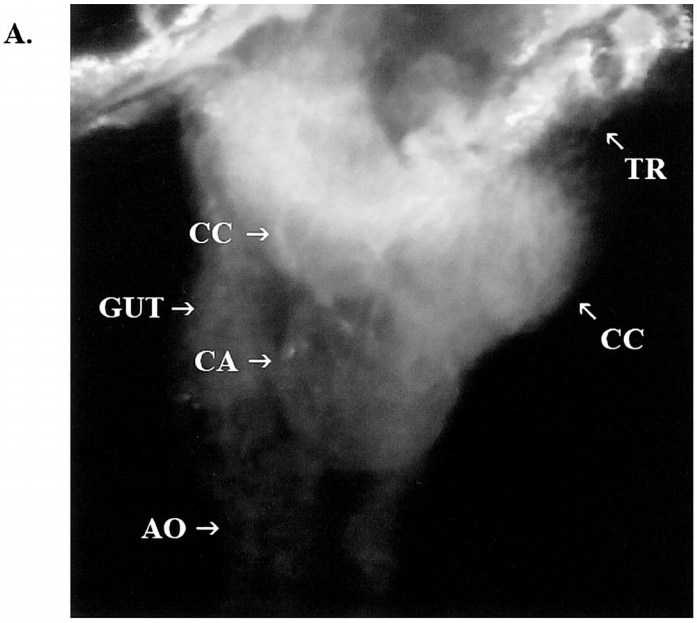


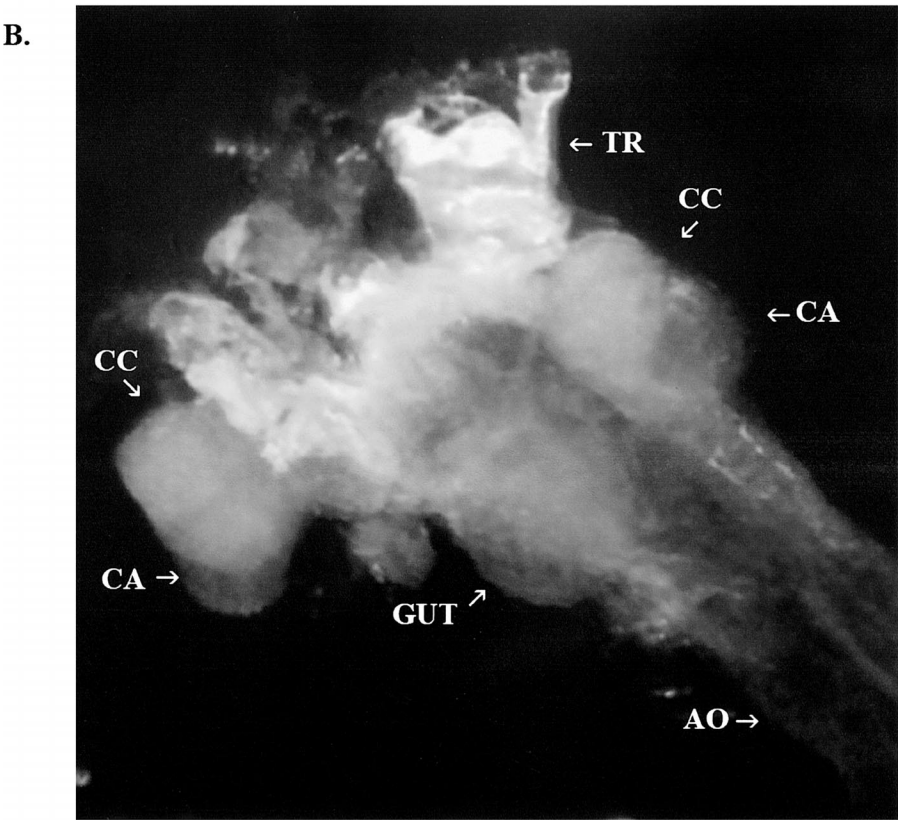


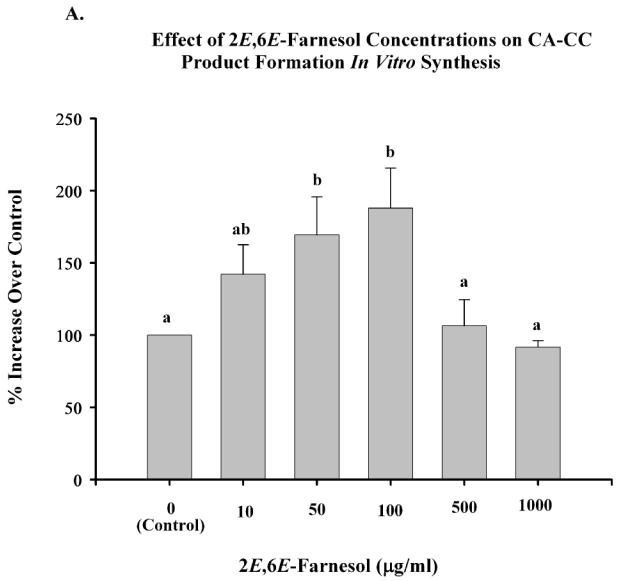
FIG. 1. Morphologies of the CA–CC complex. Note: CA, corpus allatum; CC, corpus cardiacum; AO, dorsal aorta; TR, trachea. Digital images were produced using an SZX12 stereomicroscope with an attached DP10 cooled digital camera (Olympus Co., Melville, NY). (*A*) Most common form (one CA to two CCs): one CA attached below the two CCs (×90). (*B*) Infrequent form (two CAs to two CCs): one CA attached to the periphery of each CC (×50).

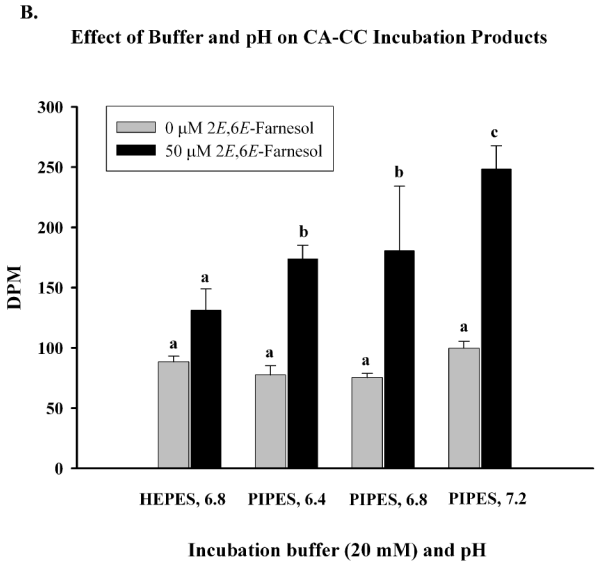
globular in shape. One fused CA is generally associated with each pair of CC (Fig. 1*A*). Nevertheless, there were occasions in which separate CA were found attached laterally to each CC, resulting in two CAs per insect (Fig. 1*B*), which also has been noted on occasion in the hemipteran *P. apterus* (M. Hodkova, pers. comm.). This latter observation is unique for hemipterans, which are generally reported as having one fused CA per complex (Richards and Davies, 1977).

To assess the metabolic function of the complex, in vitro assays were performed using a modification of the method originally developed by Pratt and Tobe (1974), and further modified by Hodkova et al. (1996), in which the incorporation of the ^{3}H-methyl group from ^{3}H-methionine into lipophilic CA products was used as a measure of the rate and extent of product synthesis. The CA–CC complexes were incubated in minimal essential medium (MEM with Hanks' salts, 20 m*M* 1,4-piperazinediethane-sulfonic acid [PIPES], pH 6.8; Sigma Chemical Co.[2], St. Louis, MO, or GIBCO-BRL, Grand Island, NY) with 5 ppm Tween 80 (15 μl per complex, Sigma,) and L-[methyl-^{3}H]-methionine (specific activity = 80–85 Ci/mmol; ARC, St. Louis, MO, or Amersham Biosciences, Piscataway, NJ) in siliconized glass tubes for 2 h, at 30° C, in total darkness. For standard assays, two complexes were incubated per tube. For thin-layer chromatography (TLC), 15 complexes were incubated per tube. After incubation, media were extracted with 100 μl isooctane per 30 μl MEM, and 20 μl extract was counted in 1.5 ml OptiPhase scintillation cocktail (Wallac/Perkin Elmer, Downer's Grove, IL) using either a Wallac 1450 or a Tri-Carb 2000CA/2100TR (Perkin Elmer) liquid scintillation counter. Chemicals used in the assays include 2*E*,6*E*-farnesol (Sigma–Aldrich, St. Louis, MO) and 3-octylthio-1,1,1-trifluoropropan-2-one (OTFP, a specific JH esterase inhibitor; a generous gift from Drs. Craig Wheelock and Bruce Hammock, University of California, Davis, CA). For incubations with 2*E*,6*E*-farnesol, stock solutions of 2 m*M* 2*E*,6*E*-farnesol were made fresh daily by vortexing on high speed for 10 min to ensure that the 2*E*,6*E*-farnesol was fully in suspension.

The optimal concentration of 2*E*,6*E*-farnesol needed to produce maximal stimulation of in vitro product synthesis was determined to be 10–100 μ*M* (Fig. 2*A*). The highest values for 2*E*,6*E*-farnesol stimulation were obtained between 1 and 3 h (percent increases over control [±SD] were: 119 ± 14% at 0.5 h; 158 ± 27% at 1 h; 264 ± 160% at 2 h; 181 ± 47% at 3 h). In addition, basal rates

[2] Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.





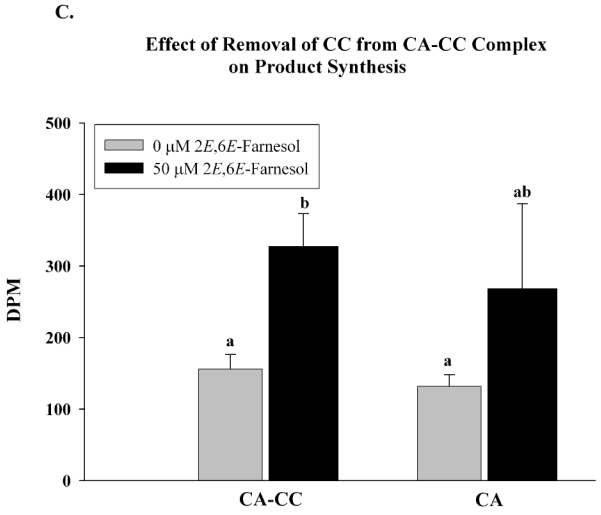


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FIG. 2. Effect of selected factors on CA–CC complex product formation. (*A*) 2*E*,6*E*-farnesol concentrations. (*B*) Buffer (20 m*M*) and pH. (*C*) Removal of CC from CA–CC complex. Two CA–CC complexes were added per tube, and the incubation was performed for 2 h at 30° C (*A* and *C*: in 1,4-piperazinediethane-sulfonic acid buffer, pH 7.2). Product was extracted with isooctane. Graph indicates percent difference from untreated control (0 μ*M* 2*E*,6*E*-farnesol) (*A*) or disintegrations per minute (dpm) (±50 μ*M* 2*E*,6*E*-farnesol) (*B*, *C*). Values represent means ± SEM from two to three separate experiments with two replications per experiment (*A*, *B*) or one experiment (n = 3) (*C*). Statistical differences were determined by analysis of variance followed by either Kruskal Wallis multiple-comparison Z-value test (*A*) or Fisher's least significant difference multiple-comparison test (*B*, *C*).

of unstimulated synthetic products did not change significantly over the time periods observed.

Studies were performed to gain a better understanding of the effect of media pH, buffer variations, and the removal of CCs from the complexes on basal rates of product synthesis and the stimulation of these rates by 2*E*,6*E*-farnesol. Two commonly used incubation buffers (PIPES and *N*-2-hydroxyethylpiperazine-*N*′-2-ethane-sulfonic acid) (Hodkova et al., 1996; Kotaki, 1996; Rachinsky and Hartfelder, 1998) were compared at different pH values. The optimal buffer and media pH for 2*E*,6*E*-farnesol stimulation was PIPES at pH 7.2, although neither buffer affected the basal rate of synthesis (Fig. 2*B*). A similar pH effect was also reported for CAs from the desert locust, *Schistocerca vaga* (Tobe and Pratt, 1974). In addition, we found that the presence of the CCs in the incubation did not affect CA activity (Fig. 2*C*), unlike Woodring and Hoffman (1997) who reported an inhibitory effect of the CCs on CA product activities because of their release of a JH esterase in crickets. This suggests that in *P. bioculatus* the CCs do not exert an inhibitory effect on CA product synthesis or hinder the stability of the CA product. Furthermore, we observed that the addition of 10 μ*M* OTFP (a JH esterase inhibitor, Hammock et al., 1984) to the CA–CC incubation medium does not enhance product stability (data not shown). These latter two observations suggest that either the CA products are not affected by potential enzymes released by the CCs or the CCs do not produce esterases that degrade JH-like CA products.

Thin-layer chromatography was performed on the medium extracts to compare the migration of the lipophilic CA product(s) (i.e., those having incorporated the ^{3}H-methyl group from the ^{3}H-methionine) to that of JHs commonly found in other insects. In these studies, the samples were spotted 1.5 cm above the bottom of a silica gel 60 F_{254} TLC plate (EM Biosciences, Gibbstown, NJ) and allowed to air dry. The plates were placed into tanks containing 10 ml solvent (either hexane:ethyl acetate [1:1], Hodkova et al., 1996, or isooctane:ethyl acetate [1:1], with no differences in results being observed between these two solvent systems). The plates were removed when the solvent front reached the top of each plate. After each run, 0.5-cm sections were placed into 2 ml OptiPhase scintillation cocktail and counted as described above. In this solvent and plate system, unlabeled JH I, II, and III were found to comigrate (as determined by ultraviolet light illumination). In addition, the extracts of the incubation medium produced a major peak (R_f = 0.77; fraction #13) that migrated slower than JH III (R_f = 0.88; fraction #15) (Fig. 3), with this peak being observed in all adults studied from age 2 to 16 d postemergence. An additional peak was found having the R_f value of 0.59 (fraction #10), although this peak was not consistently observed in all preparations and did not appear to be dependent on age or complex morphology. We also determined whether filtering (0.2 μm) the medium before extraction and TLC separation had an effect on peak formation. Unlike a previous report in which compounds unrelated to JHs were found in unfiltered media extracts (Yagi and Tobe, 2001), we found no differences between filtered and unfiltered extracts. Furthermore, the addition of 2*E*,6*E*-farnesol to the culture medium stimulated the production of the faster moving compound (R_f = 0.77), but not the slower moving compound, indicating that the former compound is more likely related to JH-like activity. Putative JHs for other hemipterans also have been reported to migrate differently from JH III using TLC (Hodkova et al., 1996; Kotaki, 1996). Our combined TLC results

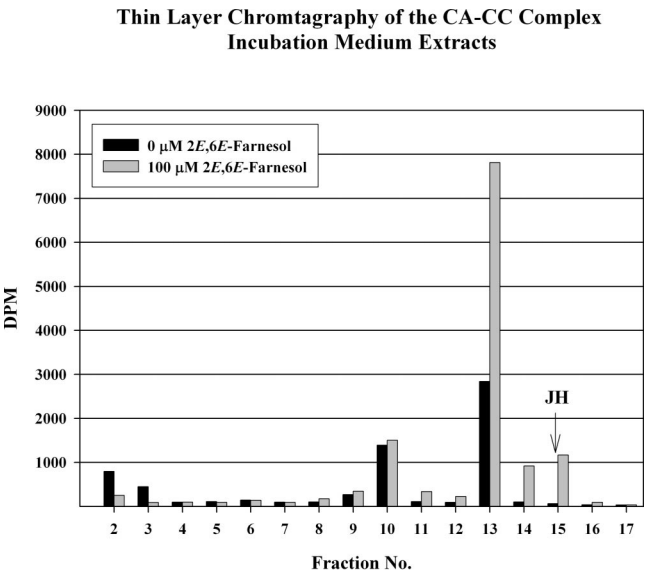

FIG. 3. Thin layer chromatography of isooctane extracts of CA–CC complex incubation medium. Incubations were performed using 15 complexes per treatment (±100 μ*M* 2*E*,6*E*-farnesol). Fractions represent 0.5-cm sections (with the origin being at 1.5 cm). The JH III standard was observable at fraction # 15. Graph indicates disintegrations per minute (dpm).

indicate that the primary lipophilic product of the *P. bioculatus* CA–CC complex is similar to that of CAs from other hemipterans.

The effects of insect age, sex, and rearing conditions on the synthesis of the CA–CC products were also studied. In these studies, no statistical differences were noted between the synthetic activities of the complexes of males and females in either their basal rates or levels of 2*E*,6*E*-farnesol stimulation (Fig. 4*A*). Similarly, no statistical differences were noted between the CA product levels in insects ranging in age from 5 to 30 d postemergence (Fig. 4*A* and *B*). Other studies have variously noted the effects of insect age and sex on the synthetic activities of the CA, with Khan et al. (1982) noting minimal effects in in vitro studies using *L. decemlineata*, and Trautmann et al. (1974a, 1974b) and McNeil et al. (2005) reporting distinct age-related effects in in vivo studies using *Tenebrio molitor* (with no JH being found in newly emerged adults) and in in vitro studies using *Pseudaletia unipuncta* (with effects varying greatly between strains), respectively. Lastly, the effect on CA–CC activity from rearing insects in conditions that would induce a diapause-like state was also tested. Insects were subjected to a decrease in light and temperature over 2 wk, from 28° C and 16-h light to 10° C and 12-h light. Under these conditions, diapause-like effects were noted, which included decreases in oviposition and respiration rates (D. G. Davis, C. L. Goodman and R. M. Wagner, pers. comm.). The CA–CC complexes from adults reared under these conditions did not respond to 2*E*,6*E*-farnesol (Fig. 4*B*). Hodkova et al. (2001) have also shown that the CAs from diapausing *P. apterus* adults undergo an inhibition of the stimulation of JH production, which they report as being due to a slow regulation mechanism.

In summary, we observed that the CA–CC complex of *P. bioculatus* has two distinct morphologies and that these complexes function similarly to the CA tissues of other insects, especially Hemipterans. In the latter case, we observed that the JH III precursor, 2*E*,6*E*-farnesol, stimulated synthesis of a lipophilic substance (Ko-

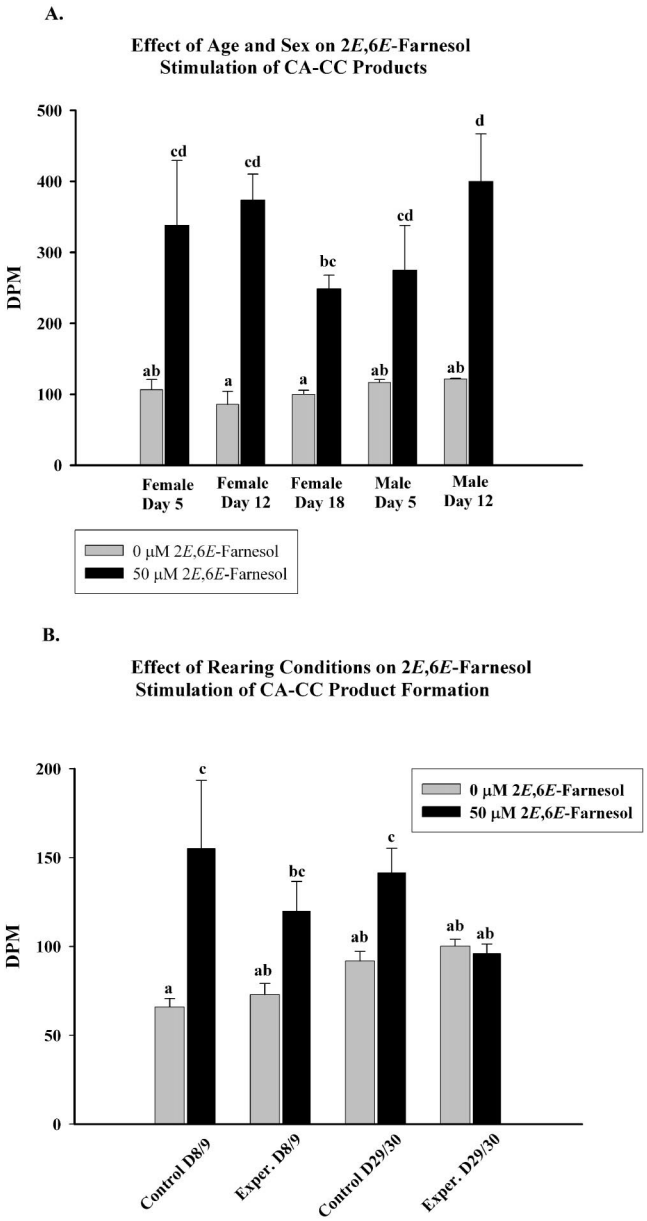

FIG. 4. Effect of age and sex of adult *Perillus bioculatus* (*A*) or subjecting female adult *P. bioculatus* to conditions inducing diapause-like behavior (*B*) on 2*E*,6*E*-farnesol stimulation of product formation by CA–CC in vitro synthesis. Incubations were performed as described in Fig. 2. For (*B*), incubations were performed on either days 8 and 9 (8/9) or days 29 and 30 (29/30) postemergence; "Cont." indicates control conditions (25° C, 16 h light); "Exper." indicates experimental conditions (from day 7 to 21 postemergence, decrease in temperature to 10° C and light to 12 h). Graph indicates disintegrations per minute (dpm) (±50 μ*M* 2*E*,6*E*-farnesol). Values represent means ± SEM (n = 2–4). Statistical differences were determined by analysis of variance followed by Fisher's least significant difference multiple-comparison test.

taki, 1996, 1997); that this substance migrated differently in TLCs than known JHs (Kotaki, 1996, 1997; Hodkova et al., 1996, 2001); and that the synthetic activities were affected by pH (Tobe and Pratt, 1974) and the metabolic state of the insect (Hodkova et al., 2001). These data will assist in our understanding of the regulation and synthesis of the putative JH from *P. bioculatus*.

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